REMARKS

The May 23, 2006 Official Action and the references cited therein have been carefully reviewed. In view of the amendments presented herewith and the following remarks, favorable reconsideration and allowance of this application are respectfully requested.

At the outset, a shortened statutory response period of three (3) months was set forth in the May 23, 2006 Official Action. Therefore, the initial due date for response is August 23, 2006.

The Examiner has maintained the objection to the August 25, 2004 Information Disclosure Statement asserting that certain references were listed but allegedly not included. Applicants maintain that copies of both the Schaeffer et al. and dos Santos et al. references were properly submitted with the Information Disclosure Statement. Indeed, Applicants have reviewed the documents listed on the Patent Application Information Retrieval system (PAIR) and note that the first reference document listed as having been submitted on August 30, 2004 contains not only the Scott et al. reference, but also the dos Santos et al. and Shaeffer et al. references attached to it. Accordingly, Applicants respectfully submit that copies of the references were clearly properly submitted with the August 25, 2004 Information Disclosure Statement and that Office error has led to the instant objection. For the Examiner's convenience, Applicants submit herewith new copies of the Schaeffer et al. and dos Santos et al. references. However, Applicants do not believe that a fee is due or a new PTO 1449 and Information Disclosure Statement is necessary in order for these references to be consideration by the Examiner because the initial IDS was properly filed with copies of these references.

The Examiner has rejected claims 1-12 and 39 under 35 U.S.C. §101 for allegedly lacking either a credible, specific

and substantial utility or a well-established utility.

Claims 1-12 and 39 have also been rejected for allegedly failing to satisfy the enablement requirement of 35 U.S.C. §112, first paragraph.

Lastly, the Examiner has rejected claim 39 under 35 U.S.C. §112, second paragraph for alleged indefiniteness.

The foregoing objection and rejections constitute all of the grounds set forth in the May 23, 2006 Official Action for refusing the present application.

No new matter has been introduced into this application by reason of any of the amendments presented herewith.

In view of the present amendment and the reasons set forth in this response, Applicants respectfully submit that the objection to the Information Disclosure Statement; the 35 U.S.C. §101 rejection of claims 1-12 and 39; the 35 U.S.C. §112, first paragraph rejection of claims 1-12 and 39; and the 35 U.S.C. §112, second paragraph rejection of claim 39, as set forth in the May 23, 2006 Official Action, cannot be maintained. These grounds of objection and rejection are, therefore, respectfully traversed.

CLAIMS 1-12 AND 39 SATISFY THE UTILITY REQUIREMENT OF 35 U.S.C. §101

The Examiner has rejected claims 1-12 and 39 under 35 U.S.C. §101 for allegedly lacking either a credible, specific and substantial utility or a well-established utility. It is the Examiner's position that none of the three utilities for the instantly claimed transgenic mice recited in the specification meet the standards for a specific and substantial utility. The three utilities cited by the Examiner are 1) the use of the transgenic mice to understand the role AHSP plays in disease processes, 2) the use of the transgenic mice to establish a non-human model for disease involving the under-expression or non-expression of AHSP, and

3) the use of the transgenic mice to identify therapeutically effective agents.

Applicants respectfully disagree with the Examiner's position. The MPEP states at §2107.02(III)(A) that "an applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement of 35 U.S.C. 101." Applicants respectfully submit that the Examiner has failed to overcome this presumption.

As stated hereinabove, the Examiner contends that the specification sets forth only three utilities for the claimed transgenic mice. This is incorrect. Claim 1 describes the aberrant phenotype from the transgenic mice of the invention. Clearly, the mice could be used to identify therapeutic agents which restore normal erythrocyte phenotype and function. Such agents may have efficacy in the treatment of certain diseases and disorders, such as thalassemias. Additionally, at pages 69 to 73, the instant specification clearly teaches that the "AHSP knock out mice of the invention can be used to advantage for the production of AHSP antibodies" (page 69, lines 28-30). Inasmuch as AHSP is "highly conserved throughout evolution," the AHSP knock out mice will allow for powerful immune responses with a more diverse array of epitope specificities to be invoked against AHSPs of various species as the AHSP administered as an immunogen will not be recognized as self in the AHSP knock out mice (see page 69, line 20 through page 70, line 13). As stated at page 70, lines 1-5, the generated antibodies can be used for "intracellular localizations, epitope mapping, and immunoprecipitation studies for characterizing those proteins that form intracellular associations with [AHSP]." Furthermore, the generated antibodies can be used to determine if an AHSP has been modified, such as by post-translational modification, and the quantity of AHSP in a sample, such as in a sample from a patient suffering from anemia or spongiform

encephalopathies (page 70, lines 14-26). The antibodies may also be used to inhibit or alter the activity of AHSP (page 70, lines 28-30).

It is also noteworthy that Example IV (pages 66-69) of the instant specification demonstrates that the loss of AHSP worsens beta thalassemia and alpha thalassemia, at least in mice. Additionally, the specification states at page 49, lines 11-15 that Miele et al. have demonstrated that AHSP is "downregulated in spleen, bone marrow, and blood of animals with transmissible spongiform encephalopathies." Accordingly, the antibodies generated via immunization of the instantly claimed transgenic mice could be used to assay a sample from an animal to determine the level of AHSP to determine if beta thalassemia and/or alpha thalassemia would be exacerbated in the tested animal or a sample from an animal to determine whether the animal has a transmissible spongiform encephalopathy.

Clearly, the instantly claimed transgenic mice are supported by the above credible, specific, and substantial utilities. Significantly, the MPEP at §2107.02(I) states that "an applicant need only make one credible assertion of specific utility for the claimed invention to satisfy 35 U.S.C. 101." Indeed, once an invention has been determined to be "useful for some purpose, it becomes unnecessary to decide whether it is in fact useful for the other purposes 'indicated' in the specification as possibly useful" (MPEP at §2107.02(I)). In light of the above-described utilities, Applicants submit that nothing more is needed to satisfy the requirements of 35 U.S.C. §101. Applicants respectfully submit that the instant rejection of claim 1-8, drawn to transgenic mice, is untenable and respectfully request its withdrawal.

With regard to methods claims 9-12 and 39, Applicants also disagree with the Examiner's assertion of a lack of

utility. Claims 9, 10, and 39 recites a method for screening for agents which affect AHSP activity by administering the agent to the instantly claimed transgenic mice and assessing for AHSP activity. Claims 11 and 12 also recite a method for assessing the activity of a compound for the treatment and/or prevention of an AHSP related disorder by administering the compound to the instantly claimed transgenic mice and assessing the characteristics of the erythrocytes.

As stated hereinabove, the loss of AHSP has been associated, for example, with transmissible spongiform encephalopathies and with the worsening of beta thalassemia and alpha thalassemia. Accordingly, it is clear that the screening for and identification of compounds which restore AHSP activity or serve as a substitute for AHSP activity possesses utility as they may be utilized to lessen the symptoms of beta thalassemia and alpha thalassemia and/or to treat transmissible spongiform encephalopathies.

Inasmuch as Applicants have clearly provided at least one credible utility for the instantly claimed methods, "utility for the claimed invention as a whole is established" (MPEP at §2107.02(I)). Nothing more is needed under 35 U.S.C. §101. In view of the foregoing, Applicants respectfully request that the instant rejection of claims 9-12 and 39 under 35 U.S.C. §101 be withdrawn.

Lastly, Applicants respectfully submit that, contrary to the Examiner's position, a skilled artisan would find the three utilities cited by the Examiner, namely the use of the transgenic mice to understand the role AHSP plays in disease processes, the use of the transgenic mice to establish a non-human model for disease involving the under-expression or non-expression of AHSP, and the use of the transgenic mice to identify therapeutically effective agents, to be specific, substantial, and well-established utilities. Indeed, as stated hereinabove, Example IV (pages 66-69) of the instant

specification teaches that the loss of AHSP worsens beta thalassemia and alpha thalassemia in mice, thereby demonstrating a role of AHSP in a disease process. The instant specification also teaches that AHSP is well conserved in different species and that the pathological features of beta thalassemia closely mimic the characteristics seen with AHSP knock out mice (see, e.g., page 63, lines 9-25; page 65, lines 19-26; and page 69, lines 20-21). Furthermore, it has been shown that the decreased expression of AHSP worsens beta thalassemia in humans (see, e.g., Galanello et al., Blood (2003) 102(11):Abstract #1881). In view of the conservation of AHSP in different species, the worsening of thalassemia in both humans and mice due to the loss of AHSP, and the similarity in clinical features of thalassemia and the the AHSP knock out mice, Applicants respectfully submit that the skilled artisan would accept the AHSP knock out mice of the instant invention as a model for disease involving the under expression of AHSP. Lastly, inasmuch as the specification demonstrates that the AHSP knock out mice are clearly a suitable model for disease and that the loss of AHSP expression is associated with a disease state, the use of the knockout mice as for the screening of therapeutic agents has a specific and substantial utility.

THE SUBJECT MATTER OF CLAIMS 1-12 AND 39 IS FULLY ENABLED BY THE DISCLOSURE IN THE SPECIFICATION AS FILED

The Examiner has rejected claims 1-12 and 39 for allegedly failing to satisfy the enablement requirement of 35 U.S.C. §112, first paragraph. Specifically, it is the Examiner's position that the specification fails to teach whether the transgenic mice of the instant invention are a model for any AHSP-associated disease or disorder. As such, the Examiner contends that the use of the claimed mice is not readily apparent and, therefore, not enabled.

Applicants respectfully disagree with the Examiner's position. The MPEP at §2107.02(I) states that "an applicant need only make one credible assertion of specific utility for the claimed invention to satisfy 35 U.S.C. 101 and 35 U.S.C. 112." For the reasons stated hereinabove, there is at least one clear, substantial utility for the instantly claimed transgenic mice and the methods of use thereof. As such, the requirements of 35 U.S.C. 101 and 35 U.S.C. 112 have been satisfied.

Additionally, Applicants note that at page 11 of the instant Official Action, the Examiner states that "nothing in the specification or the art indicates how to screen for agents that affect AHSP activity when the mice do not express AHSP." Applicants respectfully disagree. Clearly, in the instantly claimed screening methods, the test compounds or agents are screened for their ability to substitute for AHSP and restore AHSP activity. Applicants respectfully submit that the skilled artisan is fully enabled to 1) generate the instantly claimed transgenic mice (particularly in view of Example II), 2) administer a test compound to the mouse, and 3) assess the mouse for an AHSP activity, such as those recited in claim 10, or assessing erythrocytes for a characteristic as set forth in claims 11 and 39. Nothing more is required by the instantly claimed methods and, therefore, nothing more is needed to satisfy the enablement requirement of 35 U.S.C. §112, first paragraph.

In light of all the foregoing, it is respectfully submitted that the skilled person could readily make and/or use the instantly claimed invention. Accordingly, the rejection of claims 1-12 and 39 under 35 U.S.C. §112, first paragraph based on inadequate enablement is untenable and should be withdrawn.

CLAIM 39, AS AMENDED, SATISFIES THE DEFINITENESS REQUIREMENT OF 35 U.S.C. §112, SECOND PARAGRAPH

The Examiner has rejected claim 39 under 35 U.S.C. §112, second paragraph for alleged indefiniteness. It is the Examiner's position that there is insufficient antecedent basis for the phrase "said agent." Applicants have amended claim 39 to replace the phrase "said agent" with "said test compound," which has clear antecedent basis in step a) of claim 9 from which claim 39 depends. As such, Applicants respectfully request the instant rejection be withdrawn.

CONCLUSION

In view of the amendments presented herewith and the foregoing remarks, it is respectfully urged that the objection and rejections set forth in the May 23, 2006 Official Action be withdrawn and that this application be passed to issue.

In the event the Examiner is not persuaded as to the allowability of any claim, and it appears that any outstanding issues may be resolved through a telephone interview, the Examiner is requested to call the undersigned at the phone number given below.

Respectfully submitted,
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Enclosure: Galanello et al., Blood (2003) 102(11):Abstract #1881
dos Santos et al., Exper. Hematol. (2004) 32:157-162
Shaeffer et al., Biochem. (1995) 34:4015-4021

[1881] AHSP Expression in Beta-Thalassemia Carriers with Thalassemia Intermedia Phenotype. Session Type: Poster Session 52-II

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In a previous study, we described two unrelated families of Sardinian descent in wich six members, simple heterozygotes for beta thalassemia codon 39 C>T mutation at molecular analysis, showed an unusual severe phenotype of thalassemia intermedia (Gasperini et al 1998). In agreement with the hypothesis that the thalassemia intermedia phenotype in our patients may result from the interaction of the beta thalassemia mutation with another defect located outside the beta globin gene cluster, we paid attention to the recently described gene, codifying the alpha hemoglobin stabilizing protein AHSP (Kihm et al 2002). In fact this erythroid factor, by its chaperone capacity on alpha hemoglobin chains could be important in globin chain balance and therefore, if mutated, may have relevant consequences for the pathophysiology of the thalassemias. AHSP gene analysis on DNA from peripheral blood leukocytes of these patients by gene sequencing revealed no abnormality in the gene and in neighbouring 5' - 3' regions (1000 bp each region). Therefore, we have performed quantitative, real-time reverse transcriptase-polymerase chain reaction (Taqman RT-PCR) on total RNA from reticulocytes to compare the AHSP-RNA expression in these beta thalassemia carriers with thalassemia intermedia phenotype, with the expression in beta thalassemia carriers with typical hematological picture and in normal subjects. Quantitative RT-PCR was performed on a Taqman real-time PCR machine (ABI PRISM 7000, Applied Biosystems) and 18s RNA was used as control transcript to normalize quantitative data. The formula for normalization was: $2^{(-DCt)}$, with DCt representing the difference in Ct value between the target and control transcript. All expression differences were evaluated using ANOVA test. AHSP gene in these thalassemia intermedia patients was found to have a significant 9.0 fold lower average expression as compared to beta carriers and normal subjects. Similar results were obtained in eryhtroid culture grown according to Fibach method (19% of AHSP gene expression in these patients versus 100% in non beta control subjects). We may speculate that the lower AHSP gene expression could be likely responsible for the severe phenotype in our subjects. Further experiments are needed to understand the reason of the reduced AHSP expression in presence of a normal gene sequence.

Abstract #1881 appears in Blood, Volume 102, issue 11, November 16, 2003
Keywords: thalassemia|gene expression|AHSP

Sunday, December 7, 2003 5:45 PM

Poster Session: Thalassemia and Globin Gene Regulation II (5:45 PM-7:15 PM)

Degradation of Monoubiquitinated α-Globin by 26S Proteasomes†

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ABSTRACT: Ubiquitin-^{125}I-α-globin conjugate fractions containing either one (Ub_1-α), two (Ub_2-α), or a mixture of three and four ($Ub_{3,4}$-α) molecules of ubiquitin (Ub), covalently linked to one ^{125}I-α-globin molecule were isolated after incubation of a proteolysis reaction mixture containing ATP, ubiquitin aldehyde-treated reticulocyte lysate, and human ^{125}I-α-globin. Each of the purified conjugate fractions or an identically-purified control sample of unconjugated ^{125}I-α-globin was incubated as a substrate in companion proteolysis reaction mixtures containing either purified 26S or 20S rabbit reticulocyte proteasomes. The initial rate of ATP-dependent degradation of the Ub_1-α conjugate by the 26S proteasomes was ~0.44% (1.1 fmol)/min while that of the free ^{125}I-α-globin was undetectable. The initial rates of ATP-dependent degradation by the 26S proteasomes of the Ub_2-α and $Ub_{3,4}$-α conjugates were 2- to-3-fold that of the Ub_1-α species. Conversely, the degradation of free ^{125}I-α-globin and its ubiquitinated conjugates by the 20S proteasomes was not dependent on ATP, nor did it increase with the size of the Ub adduct. Analysis of the products of a reaction mixture with 26S proteasomes by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed no conversion of the Ub_1-α conjugate substrate to higher-molecular-mass conjugates. These results suggest that monoubiquitinated α-globin can be degraded significantly and specifically by interaction directly with the 26S proteasomes. This finding is consistent with the hypothesis that a substantial fraction of the Ub_1-α conjugate intermediate in the ATP-dependent proteolysis of ^{125}I-α-globin in whole reticulocyte lysate [Shaeffer, J. R. (1994) *J. Biol. Chem. 269*, 22205–22210] is degraded by this interaction.

A major pathway for the intracellular degradation of proteins, which uses ATP and ubiquitin, is present in the cytoplasm of all eukaryotes (reviewed in Hershko & Ciechanover, 1992). In this pathway, ubiquitin, a heat stable polypeptide of 8565 Da, is activated in an ATP-dependent reaction, and one or more ubiquitin molecules are subsequently linked covalently to the protein destined for proteolysis. The protein substrate moiety of these ubiquitin–protein conjugates is then degraded by the 26S proteasome, a multisubunit protease complex, in a process which also utilizes ATP (reviewed in Rechsteiner *et al.*, 1993). The 26S proteasome presumably recognizes the putative substrate for degradation by means of the catalytic ubiquitin "tag" or signal. Several investigations have shown that the structure preferred as a recognition signal for the degradation of abnormal, foreign, or short-lived natural proteins is a polyubiquitin moiety of several ubiquitin monomers (Hershko & Heller, 1985; Chau *et al.*, 1989; Hershko *et al.*, 1991). However, conjugates in which one (or a few) ubiquitin monomer(s) is attached to a long-lived natural protein have also been isolated (Goldknopf & Busch, 1977; Matsui *et al.*, 1982; Gregori *et al.*, 1985; Sokolik & Cohen, 1991; Shimogawara & Muto, 1992; Parag *et al.*, 1993); the role of these conjugates remains unknown. Recently, one of us showed that only monoubiquitinated ^{125}I-α-globin (and to a much lesser extent some diubiquitinated molecules) was observed when ^{125}I-α-globin, the protein moiety of the α chains of human hemoglobin, was incubated with unfractionated reticulocyte lysate in a proteolysis reaction mixture (Shaeffer, 1994a). Subsequent kinetic and other studies with isolated monoubiquitinated ^{125}I-α-globin during this investigation suggested that it was an intermediate in the ATP-dependent degradation of ^{125}I-α-globin. The question remained whether monoubiquitinated ^{125}I-α-globin could be recognized by the 26S proteasome or whether prior conversion to polyubiquitinated conjugates, a step undetected in the lysate reaction mixtures, was obligatory in this proteolysis. The results of the present work suggest that monoubiquitinated α-globin, but not free α-globin, can be degraded directly by purified 26S proteasomes.

MATERIALS AND METHODS

Isolation and Purification of 26S and 20S Proteasomes. The 26S proteasomes were prepared from washed rabbit reticulocytes (Green Hectares Farms, Oregon, WI). The cells were lysed with 1.5 volumes of 1 mM dithiothreitol (DTT)[1] and gentle shaking at 4 °C for 10 min. The crude lysate was made 1 mM in ATP and 5 mM in $MgCl_2$ and clarified by centrifugation at 100000*g* for 60 min. The supernatant was recentrifuged at 100000*g* for 5 h. The pellet containing

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[1] Abbreviations: DTT = dithiothreitol; SDS = sodium dodecyl sulfate; PAGE = polyacrylamide gel electrophoresis; Ub = ubiquitin; Ubal = ubiquitin aldehyde; Ub_1-α, Ub_2-α, and $Ub_{3,4}$-α = ubiquitin$_1$-^{125}I-α-globin, ubiquitin$_2$-^{125}I-α-globin, and ubiquitin$_{3,4}$-^{125}I-α-globin, i.e., the conjugates of one, two, and a mixture of three and four molecules of ubiquitin, respectively, with one molecule of ^{125}I-labeled α-globin. In this work, the conjugates can only be detected because of their ^{125}I-label, and hence, the theoretical M_r given for each (see text) includes the weight of 1 mol of ^{125}I.

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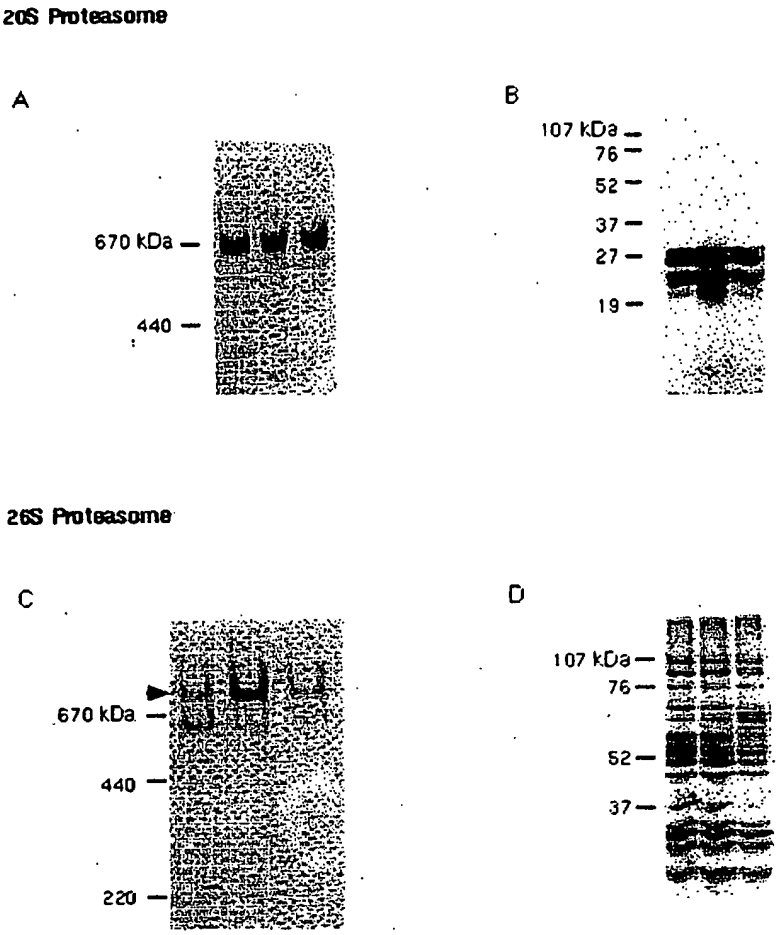

FIGURE 1: Polyacrylamide gel electrophoresis of the purified 20S and 26S proteasomes. The peak fractions of sucrose density gradients containing 20S (panels A and B) or 26S (panels C and D) proteasomes (see Materials and Methods) were resolved by 4% acrylamide nondenaturing gel electrophoresis with 50 mM Tris·HCl, pH 8.8, and 50 mM glycine buffer (A, C) or by SDS−PAGE in gels with 12.5% acrylamide (B, D) as described by Laemmli (1970). The fractions shown on the gels were pooled for use in the proteolysis reaction mixtures with ubiquitin-^{125}I-α-globin conjugates. The nondenaturing gels were stained with Coomassie Brilliant Blue, and the denaturing gels were stained with silver. An arrowhead (panel C) indicates the position of the 26S proteasomes in the nondenaturing gel. The position of the following molecular mass marker proteins, run in gel reference lanes, are shown in the figure: thyroglobulin (670 kDa), apoferritin (440 kDa), catalase (220 kDa), phosphorylase B (107 kDa), bovine serum albumin (76 kDa), ovalbumin (52 kDa), carbonic anhydrase (37 kDa), soybean trypsin inhibitor (27 kDa), and lysozyme (19 kDa); the SDS−PAGE marker proteins were prelabeled with stain and obtained from Bio-Rad Laboratories.

impure 26S proteasomes was suspended in 20 mM Tris·HCl, pH 7.6, 1 mM DTT, 5 mM $MgCl_2$, 1 mM ATP, and 10% glycerol (26S buffer) and separated by chromatography on a HiLoad 16/10 Q-Sepharose high performance column (Pharmacia No. 17-1064-01, bed volume ~20 mL) at 1 mL/min with a linear gradient of 0−500 mM NaCl in the 26S buffer. Eluted fractions which had an ATP-stimulated peptidase activity, measured with the peptide succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide as described by Tanaka *et al.* (1986), were pooled, concentrated with centrifugation filters (Amicon), and applied to a 15-mL linear gradient of 25−50% (w/v) sucrose (Peters *et al.*, 1991) in the 26S buffer without glycerol. After centrifugation in a TH641 rotor (Sorvall) at 34 000 rpm (200000g) for ~18 h, fractions separated in the sucrose gradient which had the ATP-stimulated peptidase activity were assayed for purity by nondenaturing gel electrophoresis and by SDS−PAGE (Figure 1), pooled, and frozen in aliquots at −70 °C.

The 20S proteasomes were prepared from a crude "Fraction II", precipitated with $(NH_4)_2SO_4$ as described previously (Driscoll & Goldberg, 1990), of ATP-depleted rabbit reticulocytes. The $(NH_4)_2SO_4$ pellet was suspended in and dialyzed against 20 mM Tris·HCl, pH 7.6, 1 mM DTT, and 10% glycerol (20S buffer). The dialyzed solution was separated by chromatography, as described above for the 26S particle, with a NaCl gradient in the 20S buffer. Fractions eluting at ~400 mM NaCl and containing peptidase activity (without ATP stimulation) were pooled, concentrated, and separated by centrifugation, as described above, in a 15−40% (w/v) sucrose gradient in the 20S buffer without glycerol. A gradient zone with maximal peptidase activity was harvested, assayed for purity (see Figure 1) as described for the 26S proteasomes, and frozen in aliquots at −70 °C.

Figure 1 shows protein profiles similar to those of Hough *et al.* (1987) who purified 26S and 20S proteasomes from

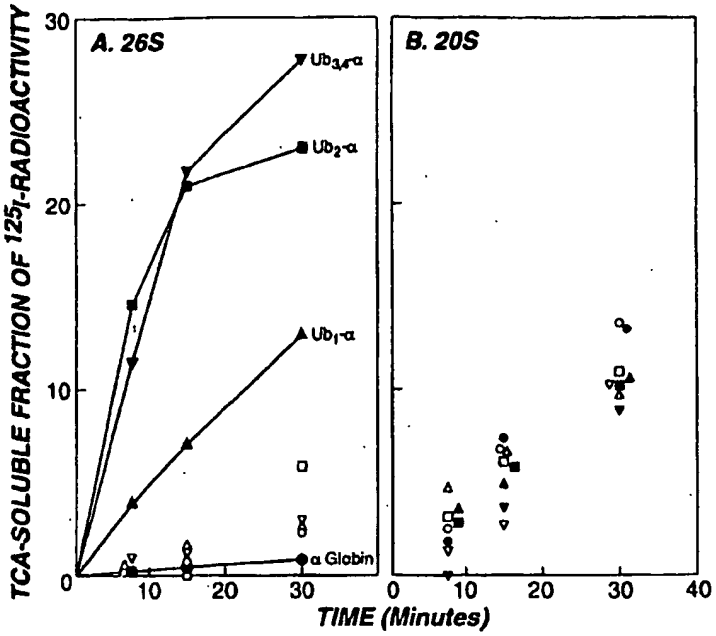

FIGURE 2: Rates of degradation of ubiquitin-^{125}I-α-globin conjugates and of free ^{125}I-α-globin in proteolysis reaction mixtures with rabbit reticulocyte 26S (panel A) or 20S (panel B) proteasomes. Replicate mixtures were prepared, incubated at 37 °C for 7.5, 15, or 30 min, and analyzed as described in Materials and Methods. The amount of proteolysis, represented by the acid-soluble fraction of the total ^{125}I-radioactivity corrected for that (≤3.0%) obtained after incubation of an identical mixture without proteasomes, is shown for the +ATP reactions by the solid symbols, as follows: Ub_1-α, ▲; Ub_2-α, ■; $Ub_{3,4}$-α, ▼; and α-globin, ●. The results from the companion −ATP reactions are shown by the corresponding open symbols.

rabbit reticulocytes by procedures employing more chromatographic steps than used by us. These profiles suggest that our preparations have a purity comparable to that of those investigators.

Construction, Incubation, and Analysis of the Proteolysis Reaction Mixture. Each mixture was prepared in a 1.5-mL plastic microcentrifuge tube and contained 50 mM Tris·HCl, pH 7.5 (23 °C), 5 mM DTT, 5 mM $MgCl_2$, 2 mM ATP *or* 0.15 unit of apyrase (Sigma No. A6410), 2.0 μg of purified 26S or 20S proteasomes, ~15% (w/v) sucrose (from the proteasome preparation), and ~800 cpm of one of the ^{125}I-labeled substrates in a total volume of 30 μL. The samples of the purified 26S or 20S proteasomes were incubated initially with 0.20 μM ubiquitin aldehyde (Ubal; Dunten & Cohen, 1989) at 37 °C for 5 min before inclusion in the reaction mixture. The ubiquitin-^{125}I-α-globin conjugate substrates (Ub_1-α, Ub_2-α, and $Ub_{3,4}$-α) were isolated by SDS−PAGE and subsequent purification procedures from a proteolysis reaction mixture containing Ubal-treated human reticulocyte lysate and human ^{125}I-α-globin, as described previously (Shaeffer, 1994a); the unconjugated ^{125}I-α-globin substrate was separately but identically exposed to SDS−PAGE and the other purification procedures. Each tube containing a proteolysis reaction mixture was capped and incubated at 37 °C for 7.5, 15, or 30 min. The reaction was stopped by chilling in ice and the addition of 200 μg (2 μL) of bovine serum albumin and 0.50 mL of 10% trichloroacetic acid (TCA). The mixtures were incubated at 4 °C for 1 h, and the acid-soluble and acid-insoluble phases were separated by centrifugation and counted in a γ-scintillation counter.

In one experiment, to examine the products of the proteolysis reaction, a 30-μL mixture containing ATP, 26S proteasomes, and 1400 cpm of purified Ub_1-α conjugate was prepared as described above. After incubation at 37 °C for 30 min, 60 μL of SDS−PAGE sample buffer (Shaeffer, 1994a) and 90 μg (0.6 μL) of human erythrocyte lysate (as carrier protein) were added, and this mixture was heated at 95 °C for 5 min. In a companion experiment, a proteolysis reaction mixture containing Ubal-treated human reticulocyte lysate, ATP, supplementary ubiquitin, and a sample of the same Ub_1-α conjugate fraction was prepared, incubated at 37 °C for 30 min, diluted with SDS−PAGE sample buffer, and heated at 95 °C, as described previously (Shaeffer, 1994a). Samples of these two diluted reaction mixtures and of duplicate mixtures not incubated at 37 °C were analyzed by SDS−PAGE (see Figure 3 legend).

RESULTS

Replicate proteolysis reaction mixtures were constructed to contain purified rabbit reticulocyte 26S proteasomes and, as a substrate, an isolated fraction of a ubiquitin-^{125}I-α-globin conjugate containing either one (Ub_1-α), two (Ub_2-α), or a mixture of three and four ($Ub_{3,4}$-α) molecules of ubiquitin covalently linked to one human ^{125}I-α-globin molecule (see Materials and Methods). Control mixtures with unconjugated ^{125}I-α-globin substrate, the precursor of the isolated conjugate fractions, were also constructed. Both conjugated and free ^{125}I-α-globin substrates had been purified identically by SDS−PAGE and ancillary procedures. The reaction mixtures were incubated at 37 °C for either 7.5, 15, or 30 min with (+) or without (−) ATP; the −ATP mixtures contained apyrase, an ATPase added to hydrolyze ATP present in the buffer of the proteasome preparations. Figure 2A shows that

the initial rate of ATP-dependent degradation, as measured by the conversion to acid-soluble ^{125}I-cpm after 7.5 min, of the Ub_1-α conjugate (▲) was ~0.44% (1.1 fmol)/min while that of the unconjugated ^{125}I-α-globin (●) was undetectable. In one experiment, the nonradioactive α-globin used as a carrier protein during the preparation of the unconjugated ^{125}I-α-globin (Shaeffer, 1994a) was replaced by ovalbumin, and the initial rate of degradation by the 26S proteasomes was still negligible; thus, the lack of proteolysis of the free ^{125}I-α-globin in Figure 2A was not due to isotope dilution. The initial rates of ATP-dependent degradation of the Ub_2-α (■) and $Ub_{3,4}$-α (▼) conjugates were 2- to 3-fold that of the Ub_1-α species (Figure 2A).

The values shown in Figure 2 have been corrected for the amounts of acid-soluble ^{125}I-cpm present in companion reaction mixtures after incubation without proteasomes. Most of this latter acid-soluble ^{125}I-cpm, which ranged from 0.5% to 3.0% of the total ^{125}I-cpm, was initially present in the unincubated substrate preparations (data not shown). Thus, the SDS−PAGE-purified ^{125}I-labeled substrates contained little apparent endogenous protease activity. The finding of an increase in the amount of ATP-stimulated proteolysis with increase in the size of the ubiquitin adduct in the substrates (Figure 2A) is consistent with the ubiquitin recognition signal or "tag" hypothesis. To investigate an alternative possibility that the increase in proteolysis observed with the higher-molecular-mass conjugate fractions was mediated by factors other than the ubiquitin moieties, e.g., non-conjugate protein contaminants copurified from the reticulocyte lysate during conjugate isolation (Shaeffer, 1994a), additional experiments were done with 20S proteasomes. The 20S proteasome is another multisubunit protease complex in the cytoplasm, which, unlike the 26S proteasome, does not prefer ubiquitinated substrates (Rechsteiner *et al.*, 1993; Waxman *et al.*, 1987; Orlowski, 1990). The degradation of free ^{125}I-α-globin and its ubiquitinated conjugates by purified rabbit reticulocyte 20S proteasomes (Figure 2B), although significant, was not dependent on ATP, nor did it increase with the amount of conjugated ubiquitin. The failure to find a pattern of substrate and energy specificity by the 20S similar to that of the 26S proteasomes is consistent with the hypothesis that the increase in ATP-dependent degradation by the latter particles of the higher-molecular-mass substrates occurs because of the ubiquitin component and its increase in size.

To explore the possibility that the Ub_1-α conjugate substrate in the experiments of Figure 2A was first converted to a polyubiquitinated conjugate by enzymes possibly contaminating the reactant preparations, a reaction mixture containing 26S proteasomes, ATP, and this substrate was incubated for 30 min, and the products were analyzed by SDS−PAGE. Little protein ^{125}I-cpm was detected in the region of the gel (Figure 3A, ●, fractions 1−28) corresponding to a range of molecular masses higher than that (23.8 kDa) of the Ub_1-α conjugate substrate. The small heterodisperse peak of protein ^{125}I-cpm at gel fractions 13−21 was also present in companion mixtures that were unincubated (Figure 3A, - - -, right-hand scale) or incubated without proteasomes (data not shown) and may represent covalently linked, nonspecific aggregates of the ^{125}I-labeled substrate. Conversely, incubation of another sample of the same Ub_1-α conjugate preparation in a reaction mixture with whole Ubal-treated reticulocyte lysate instead of purified 26S proteasomes resulted in the appearance of 31% of the initial protein ^{125}I-cpm in the higher-molecular-mass region of the gel (Figrue 3B, ●, fractions 1−28). The finding of peaks representing the Ub_2-α (32.3 kDa) and Ub_3-α (40.9 kDa) conjugate species as well as a heterodisperse zone of protein ^{125}I-cpm near the top of the gel (fractions 1−6) representing polyubiquitinated-^{125}I-α-globin (> 10^5 kDa) in this control experiment was expected because of the presence of free ubiquitin and its activating and conjugating enzymes (reviewed in Hershko & Ciechanover, 1992; Jentsch, 1992) in the whole lysate. These results suggest that, in the experiments of Figures 2A and 3A, the isolated ubiquitin$_1$-^{125}I-α-globin conjugate was degraded by interaction directly with the 26S proteasomes without prior conversion to higher-molecular-mass conjugates. The peak of product ^{125}I-radioactivity migrating at ~15 kDa (Figure 3A, ●, fractions 47−55) may represent unconjugated ^{125}I-α-globin which occurred because of Ubal-insensitive ubiquitin C-terminal hydrolase activity associated with the 26S proteasomes (Eytan *et al.*, 1993). The role, if any, of this unconjugated ^{125}I-α-globin in the pathway of the degradation of the Ub_1-α conjugate substrate by the 26S particles remains to be explored.

The relative initial rates of conversion of protein to acid-soluble ^{125}I-radioactivity of each conjugate fraction in Figure 2A presumably reflect the relative rates of degradation of the respective molecular species, because only the α-globin component is ^{125}I-labeled and each conjugate species would be expected to have the same molar specific ^{125}I-radioactivity. The specific ^{125}I-radioactivity (2.2×10^5 cpm/μg) of the unconjugated ^{125}I-α-globin progenitor of the conjugate fractions was used to estimate that each 30-μL reaction mixture initially contained ~0.25 pmol of a purified conjugate. The finding that the fraction of protein ^{125}I-radioactivity initially degraded per minute (Figure 2A) remained the same when only half of the amount of each ^{125}I-labeled substrate was included in the reaction mixture (data not shown) suggests that the initial rate of proteolysis was limited by the substrate concentration. As a precaution, in these experiments the proteasome preparations were pretreated with ubiquitin aldehyde (see Materials and Methods) to block ubiquitin−protein hydrolase activity (Hershko & Rose, 1987), a potential contaminant which might prematurely disassemble (deubiquitinate) the conjugate substrates and result in lower apparent degradation rates; however, omission of the Ubal in one experiment with Ub_1-α conjugate substrate resulted in no change in the rate of degradation. A recent report (Deveraux *et al.*, 1994) shows that short polymeric chains of ubiquitin, e.g., dimers and trimers, inhibit the binding of ubiquitin-^{125}I-lysozyme conjugates to the regulatory complex component of the 26S proteasomes. The possibility that the rates of ubiquitin-^{125}I-α-globin conjugate degradation observed in Figure 2A were influenced by the putative presence of short Ub polymers copurified during conjugate fraction isolation cannot be eliminated.

DISCUSSION

The denaturation of the ubiquitin-^{125}I-α-globin conjugate substrates by SDS during their preparation does not preclude the possibility that their specific proteolysis by purified 26S proteasomes (Figure 2A) is relevant to the degradation of similar α-globin conjugates in intact cells. The investigators (Henderson *et al.*, 1979) who developed the method of quantitative removal of SDS by treatment with triethylamine, used by us (Shaeffer, 1994a) to purify the conjugates after

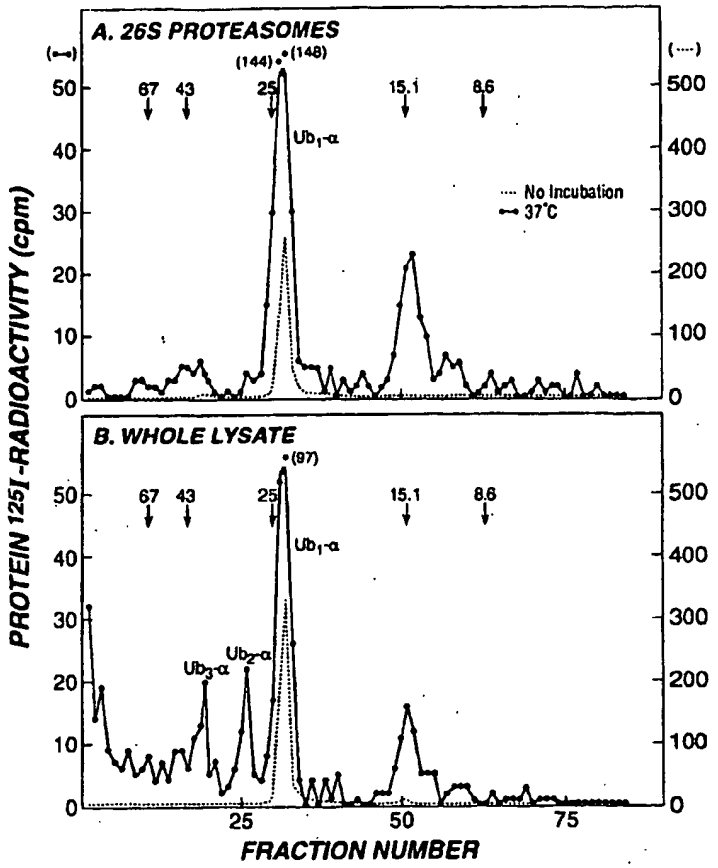

FIGURE 3: Absence of higher-molecular-mass conjugates during proteolysis of a Ub_1-α conjugate substrate by rabbit reticulocyte 26S proteasomes. Companion proteolysis reaction mixtures containing ATP, purified Ub_1-α conjugate, and either 26S proteasomes or whole reticulocyte lysate were incubated at 37 °C for 30 min and prepared for product analysis (see Materials and Methods). The figure shows the distribution (●, left-hand scale) of protein ^{125}I-radioactivity (^{125}I-cpm) after SDS−PAGE (15% acrylamide gel; Shaeffer, 1994a) of an aliquot (750 cpm) of the mixture with 26S proteasomes (panel A) and of an aliquot (840 cpm) of the mixture with whole lysate (panel B). Each panel also shows the protein ^{125}I-cpm distribution (- - -, right-hand scale) after SDS−PAGE of corresponding aliquots of duplicate reaction mixtures that were not incubated at 37 °C. The positions of molecular mass marker proteins (m = 8.6, 15.1, 25, 43, and 67 kDa; Shaeffer, 1994a) are shown in each panel. The peaks of protein ^{125}I-cpm that represent the Ub_2-α and Ub_3-α conjugate products in the reaction with whole lysate (panel B) and the Ub_1-α conjugate substrate in each mixture are denoted. The recovery of ^{125}I-cpm in the fractions separated by electrophoresis for each unincubated mixture was essentially 100% of that applied to the gel. The corresponding recoveries for the mixtures incubated at 37 °C were 83% and 68% for the reactions with 26S proteasomes or whole lysate, respectively, and were probably less than 100% because of the conversion during the proteolysis of some of the protein ^{125}I-cpm to soluble ^{125}I-cpm.

their isolation by SDS−PAGE, recovered reverse transcriptase and thromboplastin enzyme activities after exposure to this denaturant. Weber and Kuter (1971), using an entirely different method of SDS extraction, renatured a substantial fraction of aspartate transcarbamylase, aldolase, and other enzymes after SDS−PAGE. Thus, it is possible that the isolated, SDS-free conjugate substrates in this work were in a "renatured" form.

Moreover, isolated human α-globin, trace-labeled with Na^{125}I and incubated as a proteolysis substrate in a hemolysate of the erythroid blood cells from a β-thalassemic individual to produce the ubiquitin-^{125}I-α-globin conjugates (Shaeffer, 1994a), is probably similar in structure to a natural substrate in intact β-thalassemic reticulocytes. α-Globin, the apoprotein which results from removal of heme groups from hemoglobin α chains, is not usually found in the developing red cells of normal individuals. However, in β-thalassemia, a severe anemia characterized by a deficiency in the synthesis of hemoglobin β chains, a substantial excess of hemoglobin α chains accumulates in the maturing erythroid cells (reviewed in Bunn & Forget, 1986). These excess α chains are relatively unstable (Rachmilewitz *et al.*, 1971) compared to normal tetrameric ($\alpha_2\beta_2$) hemoglobin and undergo spontaneous oxidation to a methemoglobin form, some of which further denatures by heme group loss (Bunn & Jandl, 1968) to form α-globin. α-Globin has a relatively disordered secondary structure (Waks et al., 1973) compared to the α chains or tetrameric hemoglobin, and thus this "partially denatured" protein is probably a good candidate for proteolysis even without exposure to SDS.

The significant ATP-independent degradation of free ^{125}I-α-globin by purified reticulocyte 20S proteasomes (Figure 2B) but not by 26S proteasomes (Figure 2A) could suggest that nonubiquitinated proteins are degraded in intact cells primarily by the former particles. Other studies showed that casein (Tanaka et al., 1988; Driscoll & Goldberg, 1990), several oxidized, but not native, enzymes (Rivett, 1985), and lens α_2-crystallins (Ray & Harris, 1985) were degraded in

cell-free systems by 20S proteasomes from various mammalian sources. A common feature which likely renders these proteins and also α-globin in the present work particularly susceptible to cell-free proteolysis by 20S proteasomes and perhaps other proteases may be a relative lack of ordered secondary structure, as mentioned earlier. Also, the possibility that our reticulocyte 20S proteasomes, despite excellent purity (Figure 1), contained a minor contaminating protease, which spuriously degraded both ubiquitinated and free ^{125}I-α-globin, cannot be eliminated.

The above observations notwithstanding, even partially unfolded nonubiquitinated α-globin molecules may not necessarily be degraded in intact cells by free-standing 20S proteasomes. Past studies demonstrated that hemoglobin α subunits were degraded largely by the ATP- and ubiquitin-dependent proteolysis pathway in both intact β-thalassemic reticulocytes and their unfractionated lysates (Shaeffer, 1983, 1988). The present finding (Figure 2A) that purified reticulocyte 26S proteasomes selectively degrade ubiquitin-^{125}I-α-globin conjugates, shown recently (Shaeffer, 1994a) to be intermediates in the proteolysis of ^{125}I-α-globin by β-thalassemic lysates, is consistent with the earlier work and also with the hypothesis that 26S and not 20S proteasomes are involved in α-globin turnover in intact cells. Several workers showed that the 20S particle is a component of and can be incorporated into the 26S proteasome in cell-free mixtures (reviewed in Rechsteiner *et al.*, 1993). It is probable that in intact cells a large proportion of the total 20S proteasome population is present in the 26S particle (Orino *et al.*, 1991; Peters *et al.*, 1991). *In vitro* experiments in several laboratories showed that various artificial substances, e.g., SDS or polylysine, stimulate dramatically the degradation of protein or fluorogenic peptide substrates by 20S proteasomes, leading some investigators to suggest that these particles exist normally in an inactive or "latent" form (reviewed in Rivett, 1993). If so, perhaps the "activation" of the dormant 20S proteasomes in intact β-thalassemic reticulocytes occurs by the ATP-dependent assembly of the 26S complex from the 20S core particles and other protein components, a process known to occur in rabbit reticulocyte extracts (Driscoll & Goldberg, 1990; Armon et al., 1990). A determination of whether the 20S proteasome entity is directly involved in the degradation of unconjugated α-globin in intact β-thalassemic reticulocytes must await the development of a satisfactory analysis of the cellular concentration and proteolytic activity of these particles.

Rapid degradation of ubiquitin-^{125}I-lysozyme conjugates by a crude reticulocyte enzyme fraction requires the presence of a polyubiquitin moiety of several ubiquitin monomers (Hershko et al., 1984; Hough & Rechsteiner, 1986). However, conjugates of ^{125}I-lysozyme in which each of several lysine residues is ligated to a single molecule of ubiquitin modified by reductive methylation of the amino groups are also degraded at a substantial rate (Hershko & Heller, 1985). Thus, formation of polyubiquitin chains is not obligatory for the breakdown of ^{125}I-lysozyme, a relatively "foreign" protein. Other investigators (Haas *et al.*, 1990) showed that ~1% of the conjugates of ^{125}I-histone H3 ligated to reductively methylated ubiquitin could be degraded by purified 26S proteasomes during a 20-min incubation. The relevance of this observation to the proteolysis of histone, predominantly a nuclear protein, in an intact cell containing unmodified ubiquitin remains unknown. Conversely, the present results (Figure 2A) show that ~13% of an ^{125}I-α-globin substrate ligated to a single molecule of unmodified ubiquitin is degraded by the 26S proteasomes during a 30-min incubation.

In the past one of us showed that the α chains of human hemoglobin, a long-lived cytoplasmic protein, were degraded largely by the ATP- and ubiquitin-dependent proteolysis pathway in both intact reticulocytes (Shaeffer, 1983) and their unfractionated lysates (Shaeffer, 1988). This finding is consistent with those of Rock *et al.* (1994) who showed by use of peptide aldehyde inhibitors of proteasome function that most long-lived proteins inside mouse lymphoblasts were degraded by this pathway. A search for the putative conjugates of the hemoglobin α subunits when the unfractionated reticulocyte lysates were incubated with ^{125}I-α-globin (in the absence of the artificial inhibitor Ubal) revealed the presence of only the Ub_1-α (and to a much lesser extent the Ub_2-α) species (Shaeffer, 1994a). Subsequent studies showed that the Ub_1-α conjugate preparation consisted of a mixture of molecules in which 57% had Ub attached to the amino-terminal two-thirds and 43% had Ub attached to the carboxyl-terminal one-third of the ^{125}I-α-globin monomer (Shaeffer, 1994b). Both types of monoubiquitin-^{125}I-α-globin molecules were found to be intermediates in the proteolysis of nonubiquitinated ^{125}I-α-globin by unfractionated lysates. The present finding that monoubiquitinated, but not unconjugated, α-globin can be degraded at a significant rate by the 26S proteasomes suggests that polyubiquitination of free α-globin is not an obligate step in its ATP-dependent proteolysis by unfractionated lysates. These observations are consistent with the hypothesis that a monoubiquitinated molecule may be the predominant form of the conjugated intermediates in the proteolysis of hemoglobin α chains, and perhaps of other long-lived cytoplasmic proteins, in intact cells.

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Expression of α-hemoglobin stabilizing protein gene during human erythropoiesis

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α-Hemoglobin stabilizing protein (AHSP) is an abundant, erythroid-specific protein that forms a stable complex with free α-hemoglobin but not with β-hemoglobin or hemoglobin A. As such, AHSP is required for normal erythropoiesis, probably acting by blocking the deleterious effects of free α-hemoglobin precipitation. In order to study the levels of expression of the *AHSP* gene during the different phases of erythropoiesis, we carried out a two-phase liquid culture of erythroid cells and real-time quantitative polymerase chain reaction. Blood from control volunteers was cultured with erythropoietin to stimulate differentiation. The different stages of erythropoiesis were confirmed by morphologic and flow cytometric analysis. The results showed a progressive increase in *AHSP* gene expression following the expression of α-*globin* gene, during maturation of the red blood cell precursors, confirming the probable important function of this protein during normal erythropoiesis.

Miele et al. [1] described the expression of a mouse gene (named erythroid differentiation-related factor [*EDRF*]) in hematopoietic cells. Kihm et al. [2] reported that this gene produces a protein that serves as a molecular chaperone for free α-globin, providing a potential compensatory mechanism through erythroid precursors to neutralize the deleterious effects of α-globin precipitation in mouse. This protein was named α-hemoglobin stabilizing protein (AHSP).

AHSP specifically binds to the α-chains of hemoglobin but not to the β-chain or to tetrameric hemoglobin. Consistent with a role for AHSP in regulating coordinated globin expression, gene targeting studies in mice showed that ablation of AHSP function leads to erythrocyte abnormalities that also are observed in β-thalassemia [3].

When present in excess over β-globin, its normal binding partner, α-globin can have severe cytotoxic effects that are the major deleterious effects contributing to the pathophysiology of β-thalassemia. Conversely, studies in mice [3] have shown that AHSP may act as a chaperone to prevent the harmful aggregation of α-globin during normal erythroid cell development and in diseases of globin-chain imbalance. However, the expression of *AHSP* gene in humans during erythropoiesis is unknown.

In the present study, using two-phase liquid culture of erythroid cells we demonstrate a coordinate expression of AHSP and α-globin mRNA during erythroid differentiation.

Materials and methods

Two-phase liquid culture of erythroid cells

Blood from normal volunteers was cultured using a two-phase liquid culture procedure, as described in the following [4,5]. Mononuclear cells were isolated from peripheral blood samples by centrifugation on a gradient of Ficoll-Hypaque and cultured for 7 days (phase I) in IMDM medium (Gibco) supplemented with 20% fetal calf serum (Gibco), 1 μg/mL cyclosporin A (Sandoz), and 10% conditioned medium collected from bladder carcinoma 5637 cultures. Cells were incubated at 37°C in an atmosphere of 5% CO_2 and 92% humidity. After 7 days, nonadherent cells were recultured in phase II medium, IMDM supplemented with 30% fetal calf serum (Gibco), 1% deionized bovine serum albumin (BSA; Sigma), 10^{-5} M 2-mercaptoethanol (Sigma), 1.5 nmol/L glutamine (Gibco), 1 U/mL iron saturated transferrin (Sigma), 10^{-6} M dexamethasone, 5 ng/mL human stem cell factor (SCF; Calbiochem), 1 U/mL human recombinant erythropoietin (Epo; Cilag), 2.5 μg/mL Fungizone (Gibco), 50 μg/mL streptomycin (Gibco), and 25 μg/mL glutamycin (Gibco). Cell samples were collected from phase II cultures at 0, 4, 5, 7, 8, 10, 11, and 13 days to evaluate the cell numbers and viability by trypan blue exclusion for future total RNA extraction and for flow cytometric and morphologic analyses. For morphologic cell analyses, cytospin slides were prepared wet,

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stained with Leishman, and examined with an Eclipse E-600 microscopic (Nikon) using the software Image Pro-Express 4.0 (Media Cybernetic, LP) to morphologically analyze the cell differentiation stage. Expression of cell surface markers using flow cytometry was evaluated by dual staining with fluorescein isothiocyanate (FITC)-conjugated anti-transferrin receptor and phycoerythrin (PE)-conjugated anti-glycophorin A (Dako, Glostrub, Denmark) for 30 minutes at 4°C, in the dark. The cells were washed with phosphate-buffered saline and suspended in 1% paraformaldehyde prior to analysis. Data from 10,000 events were acquired for analysis using Cell Quest Software (Becton-Dickinson). Nonspecific staining was established from the corresponding isotype control and subtracted from the corresponding percentage positive population.

RNA extraction and reverse transcription

Total RNA extraction was performed using Trizol reagent (Invitrogen), a monophasic solution of phenol and guanidine isothiocyanate that improves cell lysis and dissolves cell components. Samples of 5×10^6 cells were pelleted and resuspended in 1 mL of Trizol and incubated for 5 minutes at room temperature. Chilled chloroform (0.5 mL) was added to the suspension, which was incubated for 5 minutes at room temperature and centrifuged at 13,500 rpm to achieve phase separation (organic phase from aqueous phase). The aqueous phase, containing the RNA, was transferred to a new Eppendorf tube, and RNA was recovered by precipitation with isopropyl alcohol. The RNA integrities were analyzed by electrophoreses in 1.2% denaturing agarose gel and the RNA concentration was quantified using a GeneQuant UV spectrophotometer (Pharmacia).

Five-microgram RNA samples were incubated with 1 U DNaseI (Invitrogen) for 15 minutes at room temperature, and EDTA was added to a final concentration of 2 mM to stop the reaction. The enzyme was subsequently inactivated for 10 minutes at 65°C. The DNaseI-treated RNA samples were reverse transcribed with 200 U SuperScript II (Invitrogen) for 50 minutes at 42°C. Two units of RNase H (Invitrogen) were subsequently added, and the samples were incubated at 37°C for 20 minutes. The cDNA samples were quantified using a GeneQuant UV spectrophotometer (Pharmacia).

Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) using SYBR Green dye is based on the direct detection of the PCR product, as monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA. An increased amount of PCR product will result in an increase in SYBR Green I dye fluorescence, which is collected during the course of the reaction. The resulting real-time analysis enables a more accurate quantification of nucleic acids [6–10].

Quantification of *AHSP* gene expression and α-*globin* gene expression by real-time PCR was performed with a 5700 Applied Biosystems system. Amplification of *AHSP* was performed with the Master Mix SYBR Green I reagent (Applied Biosystems). Quantification of β-*actin* expression was used as an internal control for the amount and quality of cDNA.

To quantify gene expression, a mathematical model, using calibration data ($2^{(-\Delta\Delta Ct)}$), was used. For cell culture studies, expression measured at 0 (zero) hours was used for calibration data. For this mathematical model, determination of the crossing threshold (Ct) was necessary for each transcript. Ct was defined as the point at which the fluorescence rises appreciably above the background fluorescence. The dissociation protocol was performed at the end of each run to check for nonspecific amplification. Two replicas were run on the plate for each sample, and each sample was run twice, independently.

Synthetic oligonucleotide primers were designed using Primer-Express software (Applied Biosystems) and synthesized by Invitrogen. The sequences of the primers are listed in Table 1.

All samples were assayed in duplicate in a semi-skirted PCR 96-well reaction plate (Sorenson, BioScience Inc.) with eight-strip PCR tube caps (SSI) and performed in a 25-μL volume containing 20-ng cDNA, 12.5-μL SYBR Green Master Mix PCR (Applied Biosystems), 1.7 μM *AHSP* primer, 1.2 μM β-*actin*, and 1.2 μM α-*globin* primer. To confirm accuracy and reproducibility of real-time PCR, intra-assay precision was calculated according to the equation: $E^{(-1/slope)}$, where the PCR efficiency was calculated [11,12]. The investigated transcripts showed high real-time PCR efficiency rates: *AHSP* 96.5%; α-*globin* 94.5%; and β-*actin* 100% in the investigation range of 500 ng to 2 ng cDNA input ($n = 2$) with high linearity (Pearson correlation coefficient $r > 0.95$).

Statistical analysis

The nonparametric Wilcoxon-Mann-Whitney test was used to evaluate gene expression [13].

Results

Two-phase liquid culture of erythroid cells

The culture procedure used was separated into two phases. In phase I, peripheral blood mononuclear cells were cultured for 1 week in the presence of a combination of growth factors, but without Epo. In phase II, following exposure to Epo, the cells proliferated and matured within 2 weeks into orthochromatic cells.

Cytospin preparations of cells at various stages of phase II are depicted in Figure 1A. Proerythroblasts begin to be discernible on days 3 to 4 as large, round, smooth cells that, following staining with Leishman, demonstrate deep blue cytoplasm and a large central nucleus. As these cells multiply they form clusters. During the intermediate phases, cells gradually give way to an increasing proportion of basophilic erythroblasts (days 5–7) and polychromatic and orthochromatic cells (days 8–10). In late phase II (days 11–14), erythroid cells continue to proliferate and mature into orthochromatic cells.

Table 1. Primer sequences for performance of real-time quantiative PCR

Name	Primer sequence
α-globins-RT-F	5′-TGGTCCCCACAGACTCAGAGA −3′
α-globins-RT-R	5′-CGGCCTTGA CGTTGGTCTTT −3′
β-actin-RT-F	5′-TCACCGAGCGCGGCT −3′
β-actin-RT-R	5′-TAATGTCACGCACGATTTCCC −3′
AHSP-RT-F	5′-TGATCCTCTCGTCTCTGAAGAAGAC −3′
AHSP-RT-R	5′-GCTGCCTGTAATAGTTGATGTAGAAGTT −3′

The differentiation in phase II was quantitatively determined by flow cytometry using an FITC-conjugated anti-transferrin receptor antibody and a PE-conjugated anti-glycophorin A antibody (Fig. 1B). Results demonstrated that cell differentiation toward the erythroid lineage occurred during the culture, as reflected by the increase in the percentage of transferrin receptor positive cells and percentage of glycophorin A-positive cells (0.03% and 0.11% on day 0, respectively, increasing to 65.20% and 14.94% on day 14, respectively), as previously described [4,5].

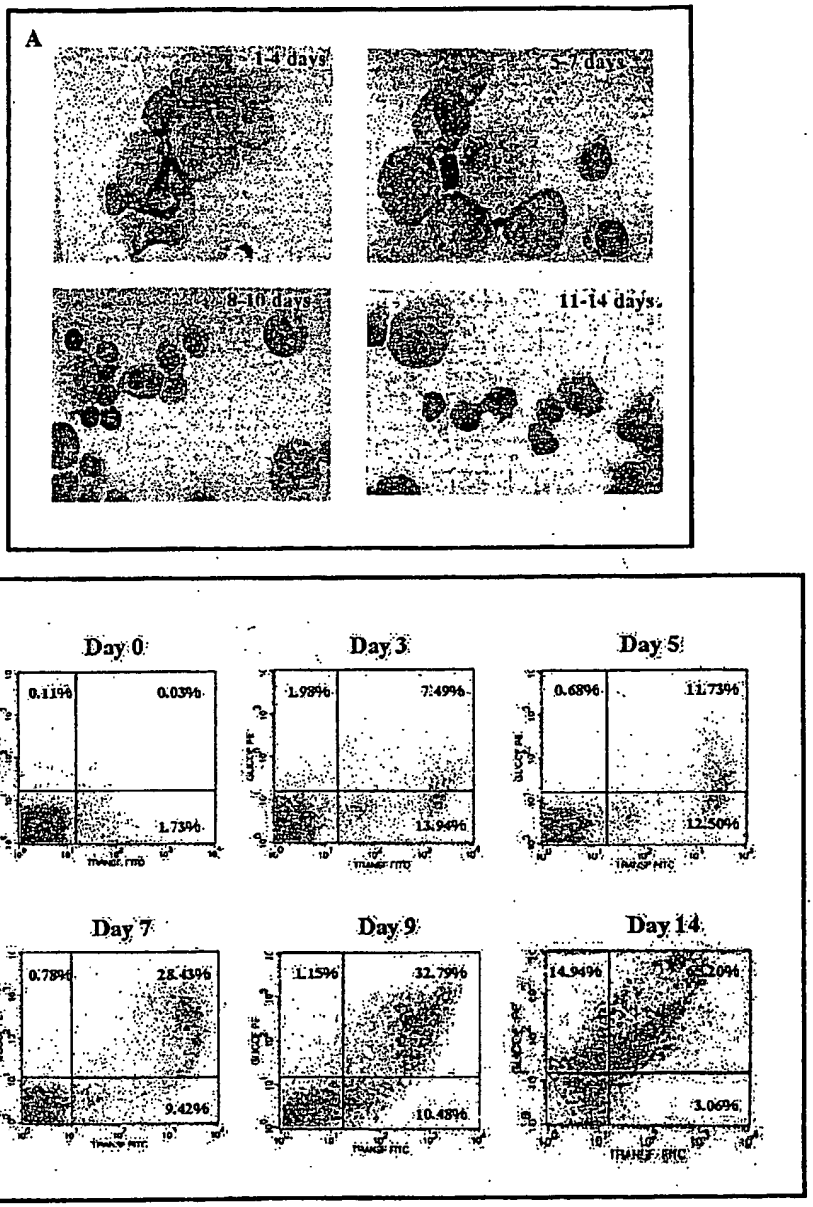


Figure 1. Two-phase liquid culture method used for culturing human erythroid precursor cells. Mononuclear cells were isolated from human peripheral blood and cultured for 7 days in phase I medium containing fetal calf serum, cyclosporin A, and growth factors. Cells were then washed and cultured in phase II medium containing erythropoietin. (A) Cells taken from phase II culture on days 0, 4, 5, 7, 8, 10, 11, and 13 (×1000) were stained with Leishman and analyzed with an Eclipse E-600 microscopic (Nikon), using the software Image Pro-Express 4.0 (Media Cybernetic, LP) to determine the cell stage by morphology. In the initial phase (days 0–4), proerythroblast cells predominate. Intermediate phases (days 5–7) demonstrate the presence of basophilic erythroblasts; On days 8–10, the presence of polychromatic and orthochromatic cells is observed. In the late phase (days 11–14), orthochromatic cells predominate. (B) Representative flow cytometric analysis of cells obtained during phase II culture. Cell differentiation was determined using FITC-conjugated anti-transferrin receptor antibody (x-axis) and PE-conjugated anti-glycophorin A antibody (y-axis). The percentage of transferrin-positive cells and glycophorin A-positive cells demonstrates the cell differentiation during phase II culture for each sample.

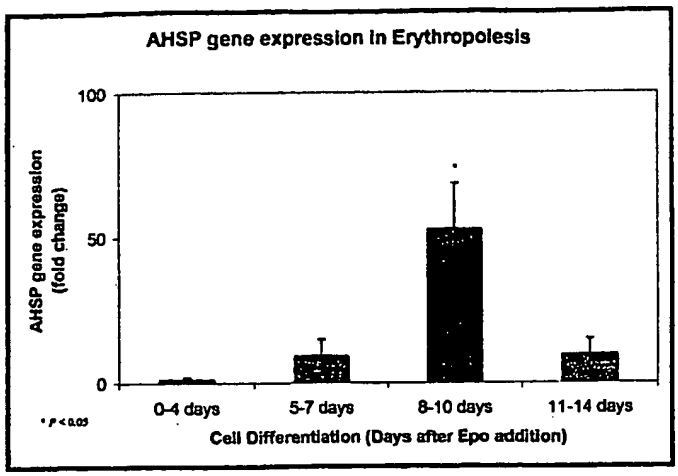


Figure 2. *AHSP* gene expression during erythroid differentiation in two-phase cell culture. The higher levels of expression ($p < 0.05$) were detected predominantly in polychromatic and orthochromatic erythroid cells (8–10 days after Epo addition). The assay was performed in a 25-μL volume containing 20-ng cDNA, 12.5-μL SYBR Green Master Mix PCR (Applied Biosystems), and the following primer concentrations: 1.7 μM *AHSP* primer and 1.2 μM β-*actin* primer.

Gene expression during erythroid differentiation in two-phase cell culture

To quantify the levels of expression during erythropoiesis, blood samples obtained from volunteers were submitted to two-phase cell culture with Epo to stimulate cell differentiation (n = 5). As described, morphologic and flow cytometric studies (Fig. 1) were used to determine differentiation stages using anti-transferrin receptor and anti-glycophorin A antibodies.

The results indicated increasing expression of the *AHSP* gene (Fig. 2) and of the α-*globin* gene (Fig. 3) during cell maturation ($p < 0.05$). The highest expression was observed during the polychromatic and orthochromatic stages (8–10 days after Epo addition), followed by a decrease in expression in more mature cells (11–14 days after Epo addition).

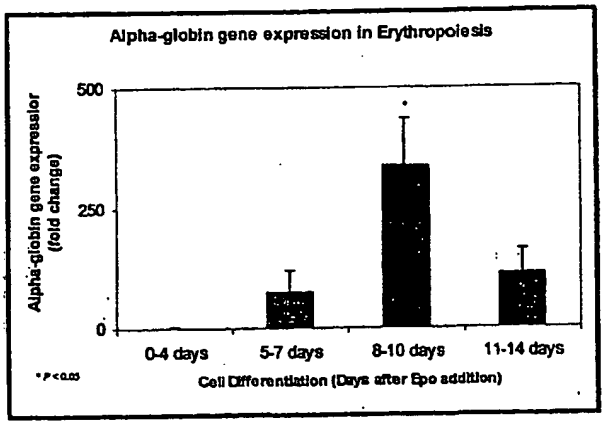


Figure 3. α-*Globin* gene expression during erythroid differentiation in two-phase cell culture. The higher levels of expression ($p = 0.05$) were detected predominantly in polychromatic and orthochromatic erythroid cells (8–10 days after Epo addition). The assay was performed in a 25-μl volume containing 20-ng cDNA, 12.5-μL SYBR Green Master Mix PCR (Applied Biosystems), and the following primer concentrations: 1.2 μM α-*globin* primer and 1.2 μM β-*actin* primer.

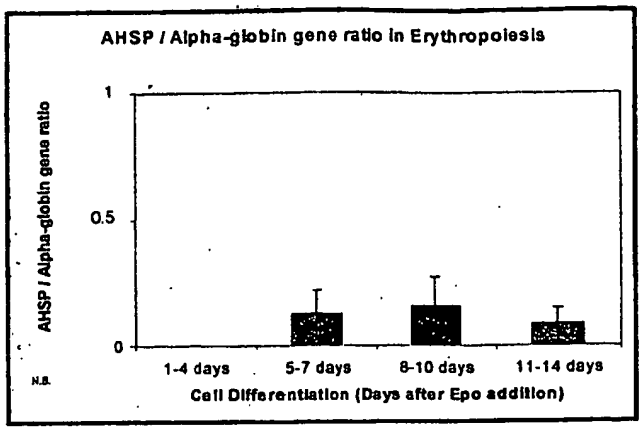
The *AHSP*/α-*globin* ratio was calculated to confirm whether there is a correlation between *AHSP* gene expression and α-*globin* gene expression (Fig. 4). The results indicated that expression of the *AHSP* gene is related to the level of expression of α-globins and that the ratio of *AHSP*/α-*globin* gene expression is similar during erythroid development, with a slight, but not significant, decrease during the later stages of differentiation ($p = 0.3$).

Discussion and Conclusion

Lower levels of β-globin production in β-thalassemia with normal synthesis of α-globin result in an imbalance in globin chain synthesis. Free α-globin chains are highly unstable and readily precipitate, damaging membrane structures and triggering the apoptotic cell death of erythroid precursors. The effect of unregulated expression of individual globin chains may be severe; consequently, it has long been thought that additional factors within the cell may assist in the processing of the free globin chains and their assembly into mature hemoglobin [14].

The identification of any such factor proved elusive for a long time. However, it has recently been shown that the *AHSP* gene is an erythroid-specific molecular chaperone for free α-hemoglobin in mice [3]. Data presented here strongly indicate that *AHSP* gene expression, in human erythroid cells, is related to α-*globin* gene expression.

To analyze the expression of *AHSP* gene during erythropoiesis, a two-phase liquid culture system was carried out



Cell Differentiation (Days after Epo addition)	*AHSP* gene expression levels (Fold Change)	*α-globin* gene expression levels (Fold Change)	Ratio ± StDev
0-4 Days	0	0	0
5-7 Days	8.9	73.6	0.12 ± 0.1
8-10 Days	52.5	336.8	0.16 ± 0.1
11-14 Days	9.3	111.1	0.08 ± 0.06

Figure 4. *AHSP* gene/ α-*globin* gene expression ratio in erythropoiesis. Graph and table show that the ratio of *AHSP* gene/α-*globin* gene was not significantly different during erythropoiesis ($p = 0.3$).

[4,5]. This technique is based on two stages. The first stage induces the proliferation of hematopoietic precursors; the second stage, following addition of Epo, stimulates erythropoiesis. Several samples were collected at differentiation to analyze gene expression during distinct cell stages of human erythropoiesis.

The pattern of *AHSP* gene expression during human erythropoiesis indicates increasing levels of expression during cell differentiation, the highest expression at the stage where hemoglobin synthesis is more intense. A reduction in expression levels occurs when the cells lose their nuclear function and hemoglobin synthesis is reduced. α-*Globin* gene expression in these cells occurred with the same pattern. Previous data clearly showed that AHSP protein binds to α-globin chains but not to β-globin chains or to tetrameric hemoglobin [2].

These findings suggest that AHSP may have an important role in normal erythropoiesis by stabilizing α-*globin*. The data presented herein indicate that, in normal erythropoiesis, the ratio of *AHSP*/α-*globin* gene expression probably is conserved during all stages of erythropoiesis. Determination of the ratio of *AHSP*/α-*globin* gene expression during all stages of erythroid development may provide a useful tool to study the possible abnormalities of the expression of this gene in hemoglobinopathies.

Most cellular proteins in eukaryotic cells are targets for degradation by the 26S proteasome, a eukaryotic ATP-dependent protease, usually after they have been covalently attached to ubiquitin in the form of a polyubiquitin chain. Proteasome functioned as a degradation signal, preventing the accumulation of abnormal protein and the formation of toxic inclusion bodies, followed by cell death [15]. Some studies have shown that an ATP-dependent system, in association with the presence of ubiquitins, is required for proteolysis of α-chains in β-thalassemia hemolysates [16,17]. AHSP is a molecular chaperone and a member of the small heat shock protein family (sHSP), probably leading free α-globins to proteolysis by an ATP-dependent protease system. Our data contribute to the hypothesis that AHSP may play an important role in the pathophysiology of β-thalassemia, decreasing ineffective erythropoiesis caused by the precipitation of free α-globin. Furthermore, the procedures described here may represent a valuable tool for study of the action of this protein as a possible additional factor interfering in the clinical evolution of these patients.

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